Vulnerabilities of transcriptome-wide association studies

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Transcriptome-wide association studies (TWAS) integrate GWAS and expression quantitative trait locus (eQTL) datasets to discover candidate causal gene-trait associations. We integrate multi-tissue expression panels and summary GWAS for LDL cholesterol and Crohn's disease to show that TWAS are highly vulnerable to discovering non-causal genes, because variants at a single GWAS hit locus are often eQTLs for multiple genes. TWAS exhibit acute instability when the tissue of the expression panel is changed: candidate causal genes that are TWAS hits in one tissue are usually no longer hits in another, due to lack of expression or strong eQTLs, even though non-causal genes at the same loci remain. Because of these vulnerabilities, it is invalid to use TWAS as a method for finding causal genes, though it can be used as a weighted burden test to identify trait-associated loci. More broadly, our results showcase limitations of using expression variation across individuals to determine causal genes at GWAS loci.

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Introduction

Transcriptome-wide association studies (TWAS) are a recent family of methods that leverage expression reference panels (eQTL cohorts with expression and genotype data) to discover associations in GWAS datasets[1,2]. TWAS begin by building predictive models of gene expression from allele counts (typically using variants within a window of 500 kb or 1 MB around the gene), then use these models to predict expression for each individual in the GWAS cohort and associate this predicted expression with the trait (Fig. 1).

TWAS have garnered substantial interest within the human genetics community and TWAS have subsequently been conducted for a wide variety of traits and tissues[3]. A key reason for the appeal of TWAS is the promise that gene-disease associations represent likely causal genes, although both papers are careful not to claim causality with absolute certainty.

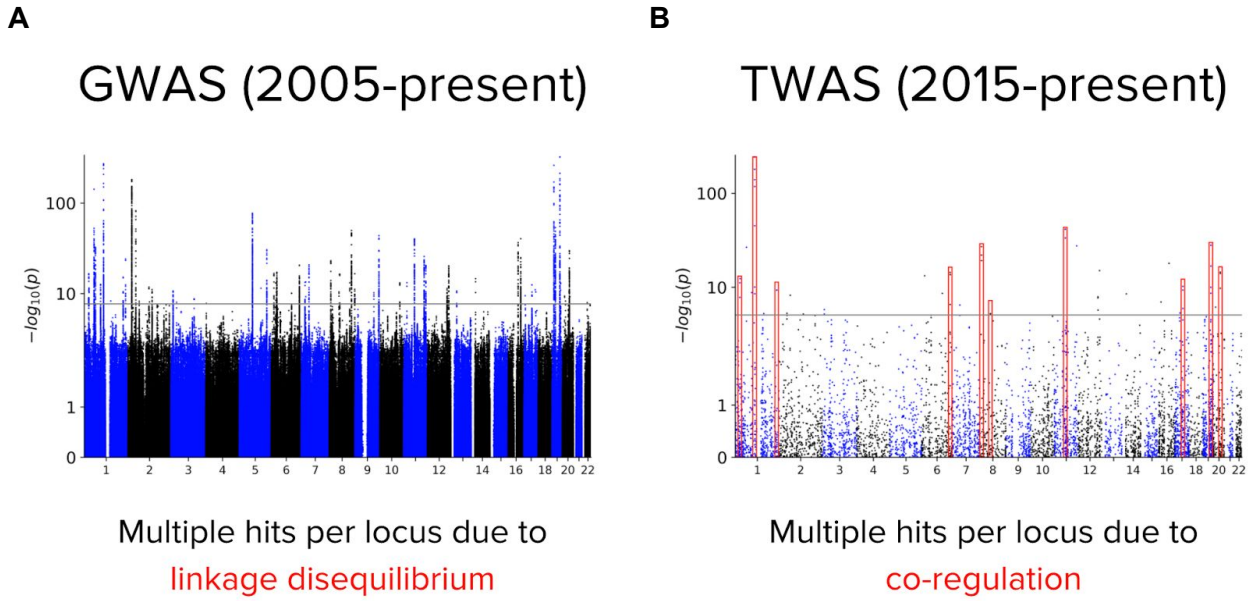
Alternatively, TWAS can be interpreted as a weighted burden test. All existing TWAS methods use a linear expression model, which means that TWAS is equivalent to testing a linear combination of variants against the phenotype, where the weights of the linear combination have been chosen based on how much the variant is predicted to contribute to expression variation across individuals in the reference panel. The goal of a weighted burden test is to increase power relative to single-variant testing (GWAS).

Results

TWAS loci frequently contain multiple hit genes

It is well known that GWAS rarely identifies single variant-trait associations, but instead identifies blocks of associated variants in linkage disequilibrium (LD) with each other (Fig. 1a). Unexpectedly, TWAS also frequently identifies multiple hit genes per locus (Fig. 1b). We call this phenomenon co-regulation.

What is the cause of this phenomenon? To answer this question, we performed TWAS in two traits and two tissues with Fusion, using GWAS summary statistics for LDL cholesterol[4] and Crohn's disease[5] and the 522 liver and 447 whole blood expression samples from the STARNET cohort[6] (Fig. S2, Online Methods). We clumped hit genes within 2.5 MB and found that while some loci contained only a single hit gene, many contained two, three, four or even up to eleven (Fig. S3).



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Scenario	Est. % of non-causal genes	Case study locus
Correlated expression	20% ($r^2 \geq 0.2$)	*SORT1* (LDL, liver)
Correlated predicted expression	75% ($r^2 \geq 0.2$)	*IRF2BP2* (LDL, liver)
Expression models share variants	69% (≥ 1 shared)	*NOD2* (Crohn's, whole blood)

Figure 1: TWAS, like GWAS, frequently has multiple hits per locus. (a), (b) Manhattan plots of GWAS and TWAS for LDL cholesterol using GWAS summary statistics from the Global Lipids Genetics Consortium and liver expression from the STARNET cohort (see Methods). GWAS has multiple hits per locus due to linkage disequilibrium, and TWAS due to a phenomenon we call co-regulation, which we explore in this paper. Clusters of multiple adjacent TWAS hit genes are highlighted in red. (c) Three scenarios where co-regulation can lead to multiple hits per locus, and the estimated percent of non-causal hit genes subject to each scenario; each scenario is presented in a case study later in the paper (a fourth scenario is presented in Fig. 5d). To estimate the percentages, we group hits into 2.5 MB clumps and make the approximation that genes that are not the top hit in multi-hit clumps are non-causal; we then calculate the percent of these genes with total or predicted expression $r^2 \geq 0.2$ or ≥ 1 shared variant with the top hit in their block, aggregating genes across the LDL/liver and Crohn's/whole blood TWAS. The full distributions of total and predicted expression correlations and number of shared variants are shown in Fig. S1, separated by study.

Correlated expression across individuals may lead to non-causal TWAS hit genes

The conventional way co-regulation is measured is by correlating the expression of a pair of genes across individuals in an expression cohort. Do genes that have correlated expression with a strong TWAS hit also tend to be TWAS hits (Fig. 5a)? We analyzed the locus containing the strongest hit gene across all four TWAS, *SORT1* in LDL/Liver (TWAS $p < 1 \times 10^{-243}$; Fig. 2a). *SORT1* has strong evidence of causality, though not without some controversy over the precise mechanism: in mouse models, overexpression of *SORT1* in liver reduced plasma LDL levels and siRNA knockdown increased plasma LDL levels[7,8], though in other studies deletion of *SORT1* counter-intuitively reduced, rather than increased, atherosclerosis in mice without affecting plasma LDL levels[9,10,11].

The *SORT1* locus contains 8 other TWAS hit genes besides *SORT1*, and their TWAS p values are highly related to their expression correlation with *SORT1* (Spearman = 0.75; Fig. 2b). Given that *SORT1* has strong evidence of causality, and that other genes at the locus lack strong literature evidence, the most parsimonious explanation is that most or all of the other genes are non-causal and are only hits due to their correlation with *SORT1*.

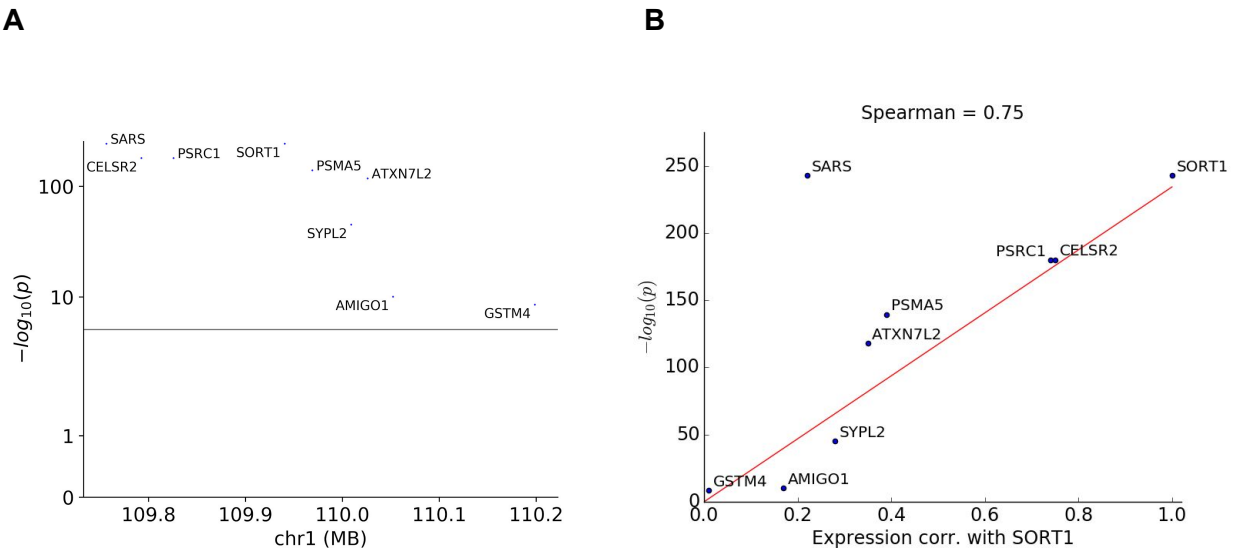

Figure 2: Co-regulation strongly predicts TWAS hit strength at the *SORT1* locus. a) TWAS Manhattan plot of the *SORT1* locus. b) Expression correlation with *SORT1* versus TWAS p value, for each gene in the *SORT1* locus.

Correlated predicted expression is sufficient for non-causal hits even without correlated total expression

However, expression correlation is not the whole story: after all, TWAS tests for association with predicted expression, not total expression. Total expression includes both genetic and environmental components, and the genetic component of expression includes contributions from common *cis* eQTLs (the only component reliably detectable in current TWAS methods),

rare *cis* eQTLs, and *trans* eQTLs. Predicted expression likely only represents a small component of the GWAS individuals' total expression: a large-scale twin study[12] found that common *cis* eQTLs explain only about 10% of genetic variation in gene expression.

While predicted expression correlations between genes at the same locus are often similar to total expression correlations, they are generally slightly higher, and sometimes substantially (Fig. 3a, Fig. S4). It is sensible for nearby genes to be more tightly co-regulated at the level of *cis* expression than at the level of total expression, since even if distinct *trans* and environmental effects act on the two genes, they do at least share the same *cis* sequence context.

Predicted expression correlation may lead to non-causal hits even for genes with low total expression correlation (Fig. 5b). For instance, *SARS* is the main outlier in Fig. 2b because, despite having a similar TWAS p value to *SORT1*, it has an unexpectedly low expression correlation of approximately 0.2; yet it is still a strong hit because of its high predicted expression correlation of approximately 0.9 (Fig. 3a).

Another example is the *IRF2BP2* locus in LDL/liver (Fig. 3b), where *RP4-781K5.7* is a likely non-causal hit due to predicted expression correlation with *IRF2BP2*, a gene encoding an inflammation-suppressing regulatory factor with strong evidence of causality from mouse models, at least at the level of atherosclerosis[13]. While there is almost no correlation in total expression between the two genes (Pearson = -0.02), *IRF2BP2*'s expression model includes a GWAS hit variant, rs556107, with a negative weight while *RP4-781K5.7*'s includes the same variant, as well as two other linked variants, with positive weights (Fig. 3c), resulting in almost perfectly anti-correlated predicted expression between the two genes (Pearson = -0.94).

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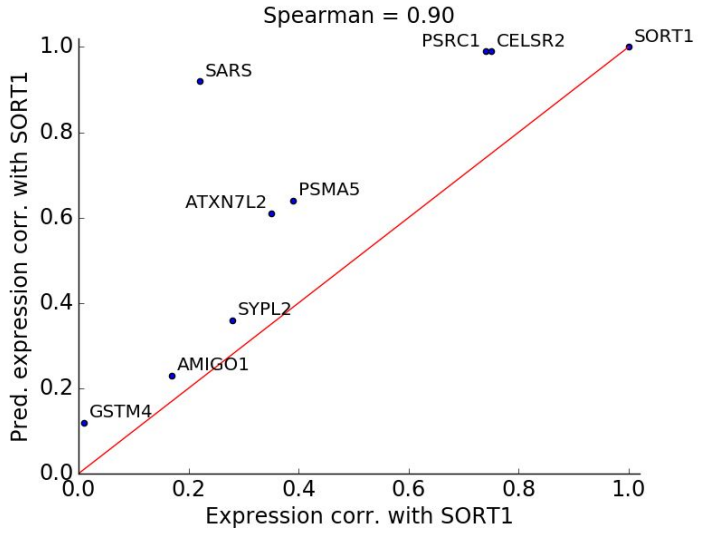

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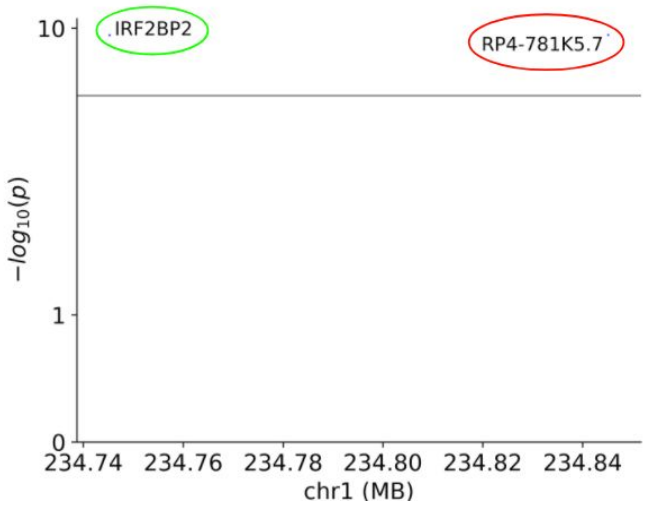

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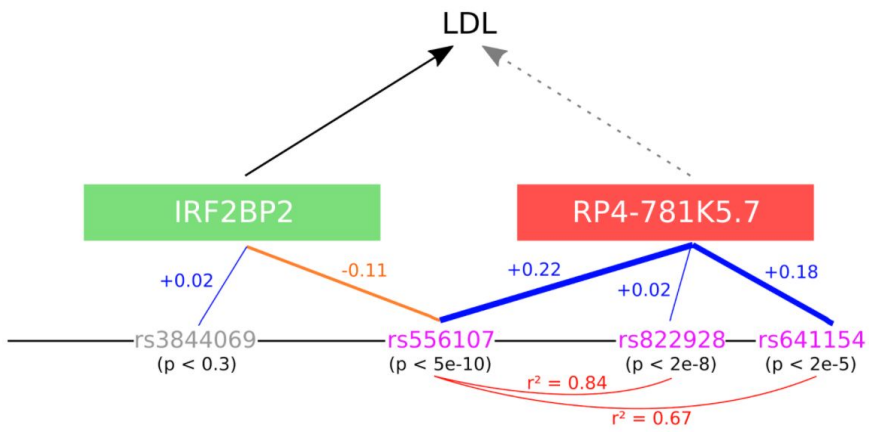

Figure 3: Correlated predicted expression can cause non-causal hits even in the absence of correlated total expression. a) Predicted expression correlations tend to be higher than total expression correlations, e.g. at the *SORT1* locus. b) TWAS Manhattan plot of the *IRF2BP2* locus, where *RP4-781K5.7* is a likely non-causal hit due to predicted expression correlation with *IRF2BP2*. c) Details of the two genes' expression models: a line between a variant's rs number and a gene indicates the variant is included in the gene's expression model with either a positive weight (blue) or negative weight (orange), with the thickness of the line increasing with the magnitude of the weight; red arcs indicate LD. Pink rs numbers are GWAS hits (genome-wide-significant or sub-significant) while gray rs numbers are not.

<u>Shared GWAS variants can cause non-causal hits even without correlated predicted expression</u>

More generally, pairs of genes may share GWAS variants in their models even if they have low predicted expression correlation, since other variants that are distinct between the models may "dilute" the correlation (Fig. 5c). For instance, at the *NOD2* locus for Crohn's/whole blood, *NOD2* is a known causal gene[14,15], but 4 other genes are also TWAS hits (Fig. 4a), none with

strong evidence of causality (though rare variants in one gene, *ADCY7*, have been associated with ulcerative colitis but not Crohn's[16]). The model for the strongest hit at the locus, *BRD7*, puts most of its weight on rs1872691, which is also the strongest GWAS variant in *NOD2*'s model (Fig. 4b). However, the *NOD2* model puts most of its weight on two other variants, rs7202124 and rs1981760, which are slightly weaker GWAS hits. The result is that even though there is information sharing between the models, and *BRD7* appears to be a non-causal hit because its model uses a variant that likely derives its GWAS signal from *NOD2*, the overall predicted expression correlation between the two genes is very low (-0.03), as is the total expression correlation (0.05).

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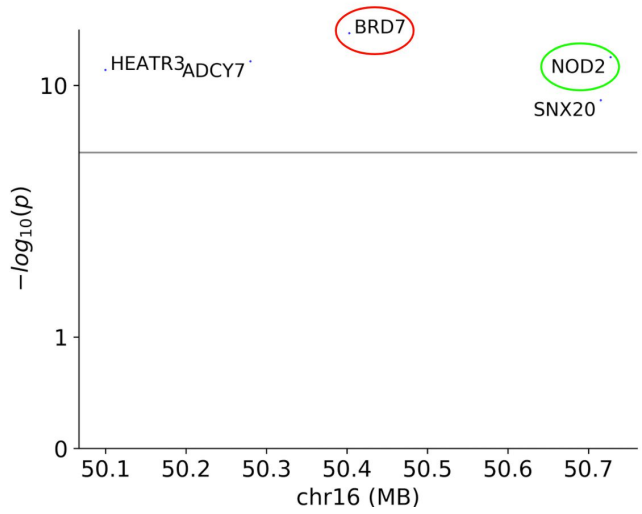

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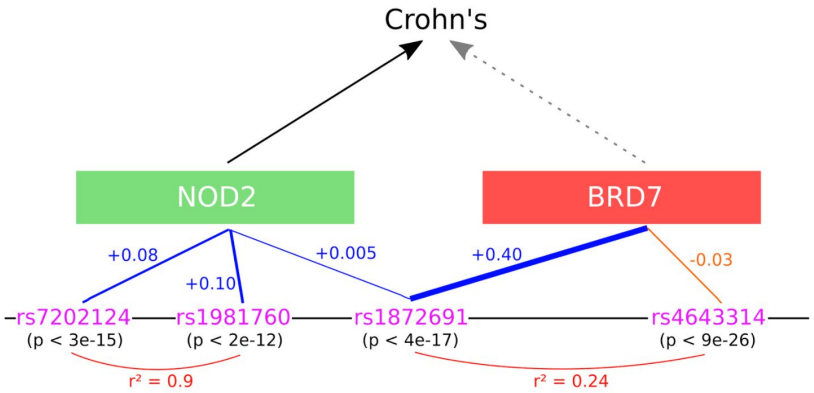

Figure 4: Sharing of GWAS variants between expression models can contribute to non-causal hits even without correlated predicted expression. a) TWAS Manhattan plot of the *NOD2* locus. b) Details of the expression models of *NOD2* and *BRD7*: as in Fig. 3, a line between a variant's rs number and a gene indicates the variant is included in the gene's expression model with either a positive weight (blue) or negative weight (orange), with the thickness of the line increasing with the magnitude of the weight; red arcs indicate LD.

In the most general case, models need not even share the same GWAS variants for there to be non-causal hits (Fig. 5d). For instance, the other two variants in *NOD2*'s model are neither shared nor in strong LD with of the variants in *BRD7*'s model (Fig. 4b). Under the assumption that *NOD2* is the only causal gene at the locus, this suggests that these variants are GWAS hits because they (or variants in LD) regulate *NOD2* as well as *BRD7*, but that this connection is missed by *NOD2*'s model, i.e. the expression modeling has a false negative. This type of scenario might occur even without any false negatives in the expression modeling, e.g. if the two *NOD2* variants (or variants in LD) deleteriously affected the coding sequence of *NOD2* as well as regulating *BRD7*.

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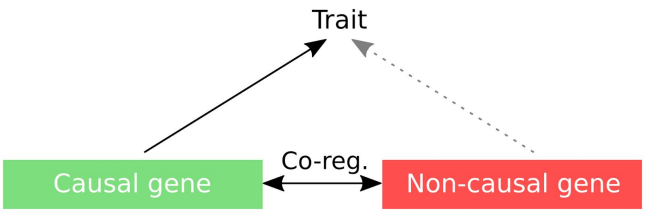

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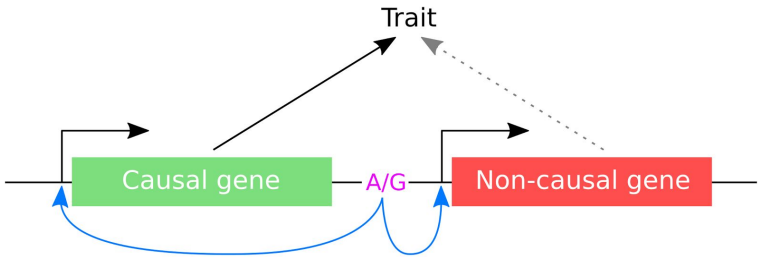

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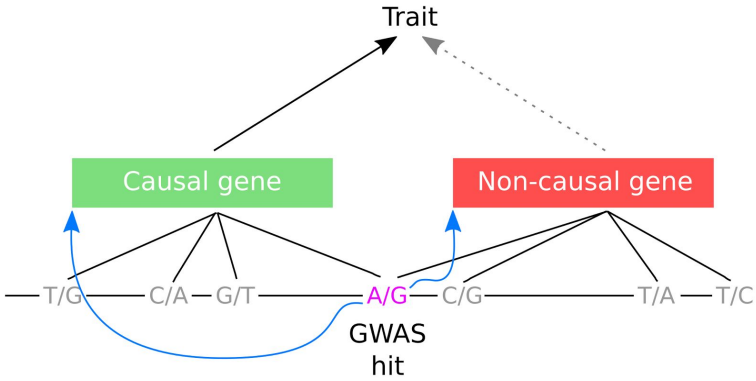

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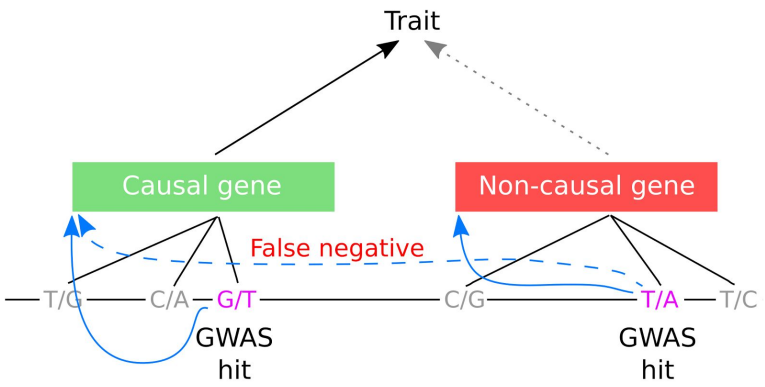

Figure 5: Co-regulation scenarios in TWAS that may lead to non-causal hits, from least to most general. a) Correlated expression across individuals: the causal gene has correlated expression with another gene, which may become a non-causal TWAS hit. b) Correlated predicted expression across individuals: even if total expression correlation is low, predicted expression correlation may be high if the same variants (or variants in LD) regulate both genes and are included in both models. c) Sharing of GWAS hits: even if the two genes' models include largely distinct variants and predicted expression correlation is low, only a single shared GWAS hit variant (or variant in LD) is necessary for both genes to be TWAS hits. d) Both models include distinct GWAS hits: in the most general case, the GWAS hits driving the signal at the two genes may not be in LD with each other, for instance if the non-causal gene's GWAS hit happens to regulate the causal gene as well but this connection is missed by the expression modeling (a false negative), or if the causal gene's GWAS hit acts via a coding mechanism (not shown).

<u>Using expression from less related tissues substantially worsens the effects of co-regulation</u>

So far, all our TWAS case studies have used expression from tissues with a clear mechanistic relationship to the trait: liver for LDL and whole blood for Crohn's. What if we swap these tissues (liver for Crohn's and whole blood for LDL), so that we are using tissues without a clear mechanistic relationship? It is well-known that the architecture of eQTLs differs substantially across tissues: even among strong eQTLs in GTEx ($p \sim 1 \times 10^{-10}$), one quarter switch which gene they are most significantly associated with across tissues[17].

We manually curated causal genes from the literature at 9 LDL/liver and 4 Crohn's/whole blood multi-hit TWAS loci and looked at how their hit strengths changed when swapping tissues (Fig. 6). Strikingly, almost every candidate causal gene (9 of 11 for LDL and 5 of 6 for Crohn's) was no longer a hit in the "opposite" tissue, either because they were not sufficiently expressed (N = 4: *PPARG*, *LPA*, *LPIN3*, *SLC22A4*) or because they did not have sufficiently heritable *cis* expression, according to a likelihood ratio test, to be tested by Fusion (N = 10: *SORT1*, *IRF2BP2*, *TNKS*, *FADS3*, *ALDH2*, *KPNB1*, *SLC22A5*, *IRF1*, *CARD9*, *STAT3*).

Worse, 15 other genes at the same loci were still hits (8 in LDL/whole blood and 7 in Crohn's/liver), and 5 were even strong hits with $p < 1 \times 10^{-20}$. This suggests that the strategy of conducting TWAS in a tissue that is sub-optimal for the trait being examined (e.g. whole blood, lymphoblastoid cell lines), just because that tissue happens to have a large expression

reference panel, is especially problematic because many hit loci may contain only non-causal genes and the causal gene may not even be included in the list of hits.

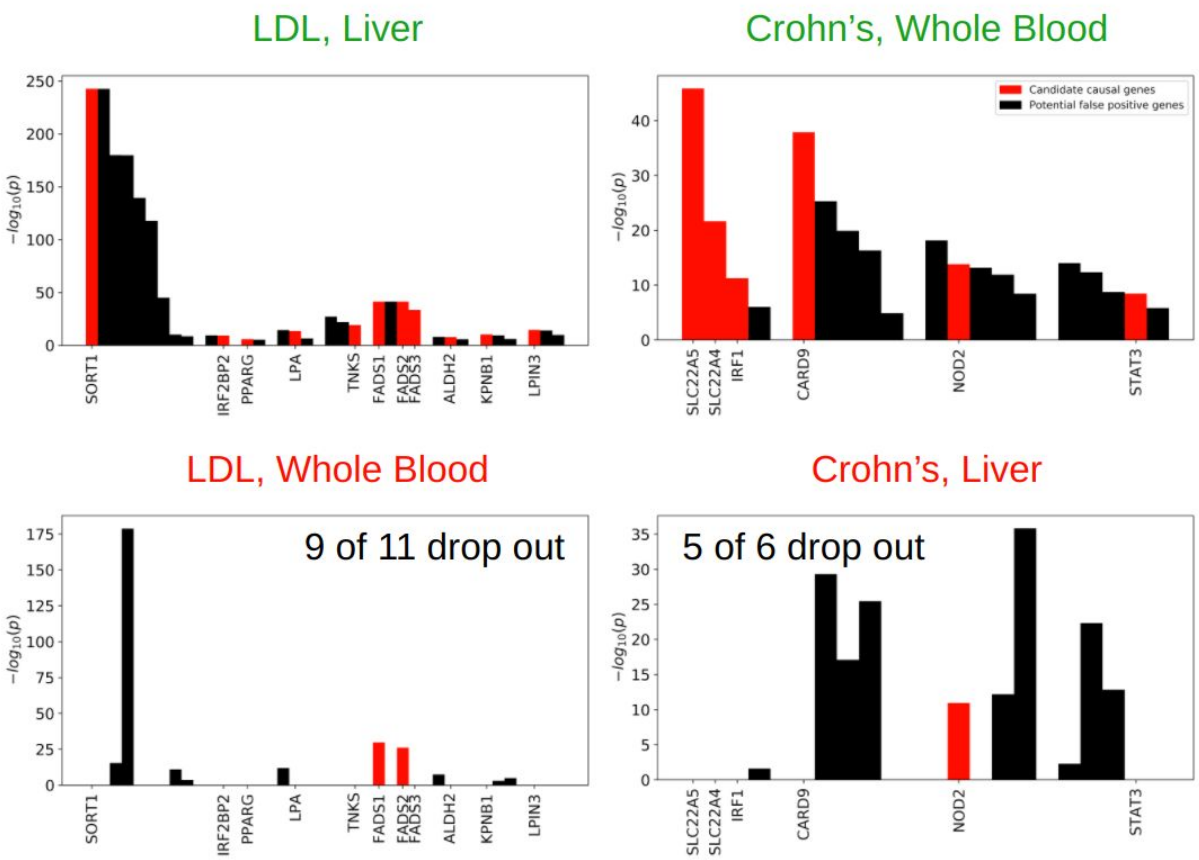

Figure 6: Most candidate causal genes drop out when switching to a tissue with a less clear mechanistic relationship to the trait, due to lack of sufficient expression or sufficiently heritable expression. TWAS p values at 9 LDL/liver and 4 Crohn's/whole blood multi-hit loci, when using expression from tissues with a clear (top row) and less clear or absent (bottom row) mechanistic relationship to the trait. Candidate causal genes are labeled and colored in red.

Discussion

We have shown that it is invalid to use TWAS as a method for finding causal genes, since it is highly vulnerable to non-causal gene-trait associations, intuitively because GWAS hits may be eQTLs for multiple genes. However, the ways in which co-regulation may lead to non-causal hits in TWAS are multi-faceted; co-regulation is hard to quantify, let alone correct for. The problem is particularly acute when using expression from tissues without a clear mechanistic relationship to the trait. It is still valid to use TWAS as a weighted burden test, where the goal is not to identify causal genes but merely discover associated loci.

Is it possible, despite the limitations of TWAS, to somehow perform statistical fine-mapping and determine the causal gene or genes? We believe that it is not, even in principle. This is

because predicted expression only imperfectly captures *cis* expression, the component of expression driven by variants near the gene; there are sources of both variance and bias in the expression modeling. The main source of variance is the finite size of the reference panel, although this can be mitigated with resampling methods. More problematically, the choice of tissue, cell type composition and quantification of the expression panel can all introduce bias. We have shown that using a tissue with a less clear mechanistic relationship to the trait hinders the ability to detect most candidate causal genes. Yet diseases rarely act through a single tissue: different genes may be causal in different tissues, so even using a tissue where most genes are causal may introduce bias for the remaining genes that are causal in a different tissue. Furthermore, most expression panels are gathered for tissues, not cell types, and genes may only be causal for a single cell type within a tissue. There may be substantial cell type heterogeneity within and between samples (e.g. due to the presence of blood and immune cells), which can also introduce bias. It is impossible to quantify every source of bias.

In our case studies, we have generally assumed that the single gene with substantial evidence of causality is the sole causal gene at the locus, with some exceptions where there are multiple candidates and the causal gene or genes are under debate (*FADS1-3*, *SLC22A4*/*5*/*IRF1*). While this is the most parsimonious explanation, it is possible that some loci harbor multiple causal genes. Indeed, under an omnigenic model of complex traits[18], every gene may be causal to some degree. Furthermore, the expression of other genes at the locus may causally contribute to the expression of the causal gene, merely by being actively transcribed, even if the gene is non-coding or its protein product has no causal role[19].

The vulnerabilities we have identified in TWAS, co-regulation and tissue bias, also apply to other methods that integrate GWAS and expression data. Gene-trait association testing based on Mendelian Randomization (MR)[20,21,22] is vulnerable to non-causal hits because co-regulation, as a form of pleiotropy, violates one of the core assumptions of MR[23]. While the HEIDI test[20] is designed to correct MR in the case where the two genes have distinct, but linked, causal variants, it does not control for the case where the two genes share the same causal variant. GWAS-eQTL colocalization methods such as Sherlock[24], coloc[25,26], QTLMatch[27], eCaviar[28], enloc[29] and RTC[30] are also vulnerable to this phenomenon. The more tightly a pair of genes is co-regulated in *cis*, the more difficult it becomes to distinguish causality based on GWAS and expression data alone. Our results underscore the need for computational and experimental methods that move beyond using expression variation across individuals to determine the causal genes at GWAS loci.

Methods

TWAS were performed with the Fusion software (https://github.com/gusevlab/fusion_twas/tree/9142723485b38610695cea4e7ebb508945ec006c), using default settings and also including polygenic risk score as a possible model during cross-validation in addition to BLUP, Lasso, and ElasticNet. Variants in the STARNET reference panel were filtered for quality control using PLINK[31] with the options "--maf 1e-10

--hwe 1e-6 midp --geno". STARNET expression was processed as described in the STARNET paper[6], including probabilistic estimation of expression residuals[32] (PEER) covariate correction. Because Fusion, to our knowledge, only supports training on PLINK version 1 hard-call genotype files and not genotype dosages, we trained expression models on only the variants both genotyped in STARNET and either genotyped or imputed in the GWAS, filtering out variants without matching strands between the GWAS and STARNET. Expression models were trained on all remaining variants within 500 kb of a gene's TSS, using Ensembl v87 TSS annotations for hg19[33]. Linkage disequilibrium and total and predicted expression correlations were calculated across individuals in STARNET. Code to replicate the post-TWAS analysis is available at https://github.com/Wainberg/Vulnerabilities_of_TWAS.

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Author Contributions

M.W., M.R. and A.K. conceived of the study. M.W. performed analyses. N.S.-A., D.K. and D.G. provided intellectual input. R.E., A.R., T.Q., K.H. and J.B. provided assistance with the STARNET dataset. M.R. and A.K. supervised the study. M.W., M.R. and A.K. wrote the manuscript. All authors reviewed the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

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Supplementary Information

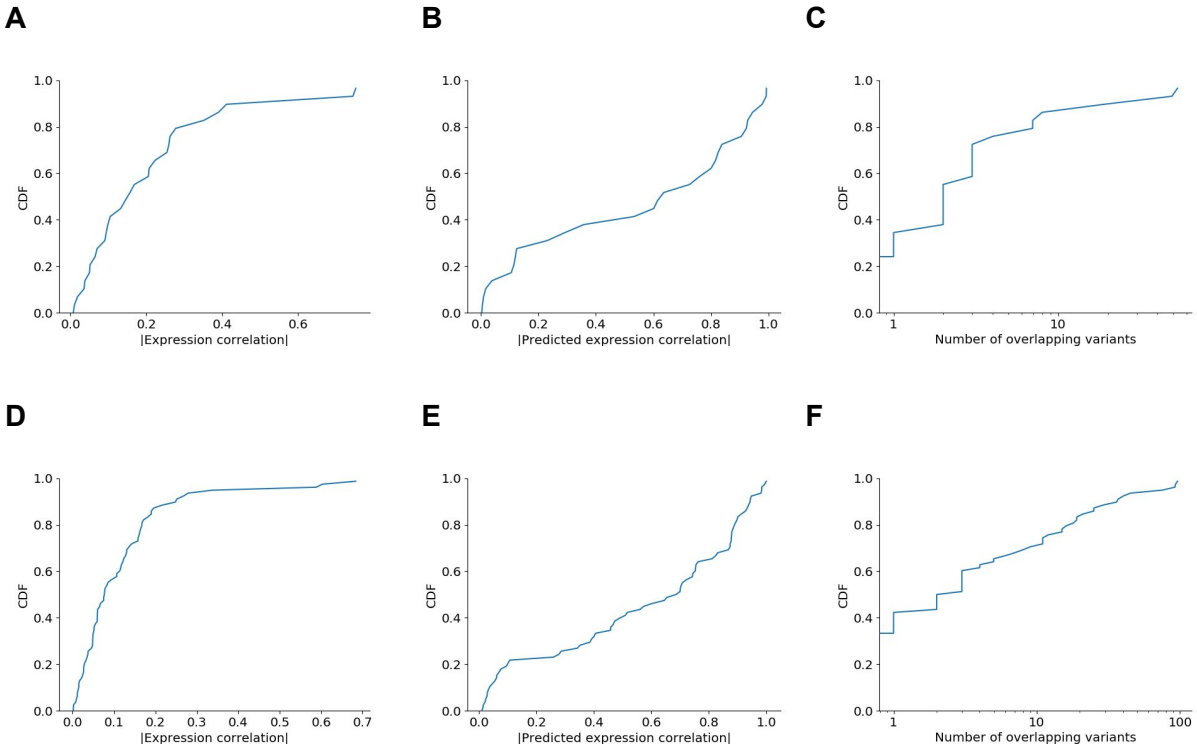

Figure S1: Distributions of co-regulation across putative non-causal genes in multi-hit TWAS loci. Since many multi-hit loci do not have a clear causal gene or have multiple plausible candidates, we make the approximation that only the most significant gene at each locus is causal. We then plot the cumulative distribution functions (CDFs) of (a, d) expression correlations, (b, e) predicted expression correlations and (c, f) number of shared variants between these most significant genes and all the other genes at their loci, separately for LDL/liver (a-c) and Crohn's/whole blood (d-f). To collapse these CDFs into a single estimate of the percent of affected non-causal genes (Fig. 1c), we combine genes across the two studies and threshold to correlation $r^2 \geq 0.2$, a threshold commonly used for weak LD in GWAS, or ≥ 1 shared variant. Note that counting only exact sharing of variants does not account for LD, for simplicity.

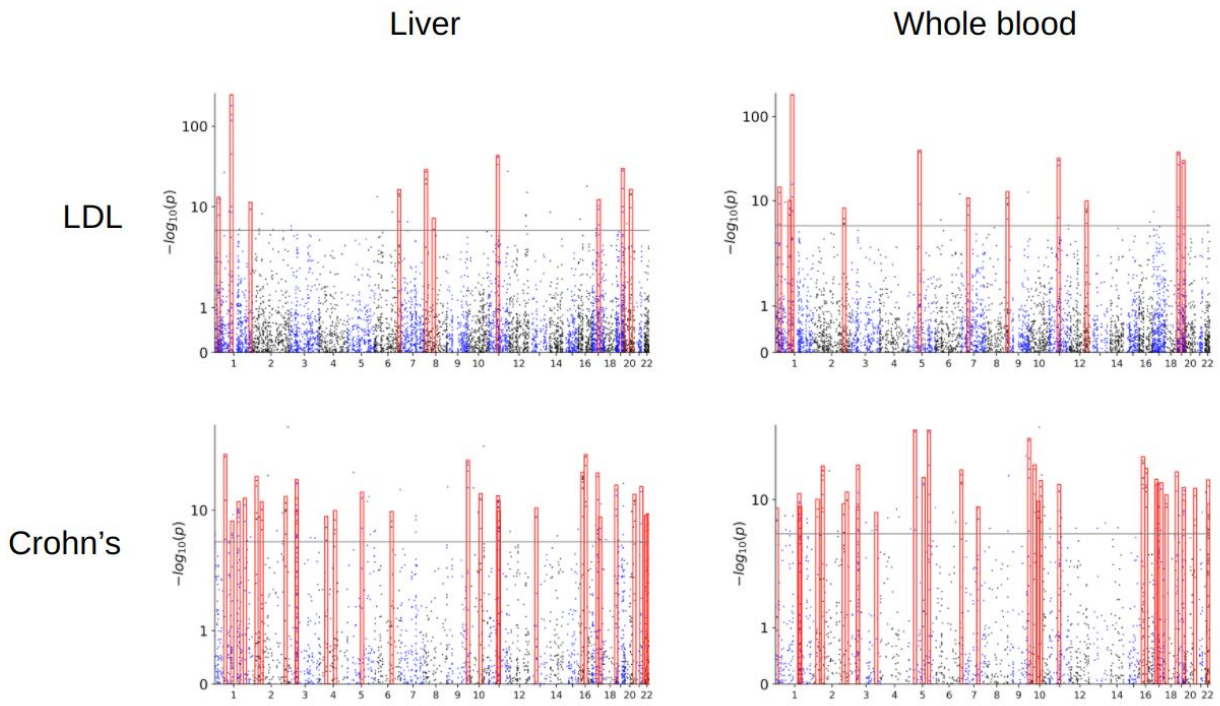

Figure S2: Manhattan plots of the 4 TWAS conducted in this study. As in Fig. 1, clusters of multiple adjacent TWAS hit genes are highlighted in red.

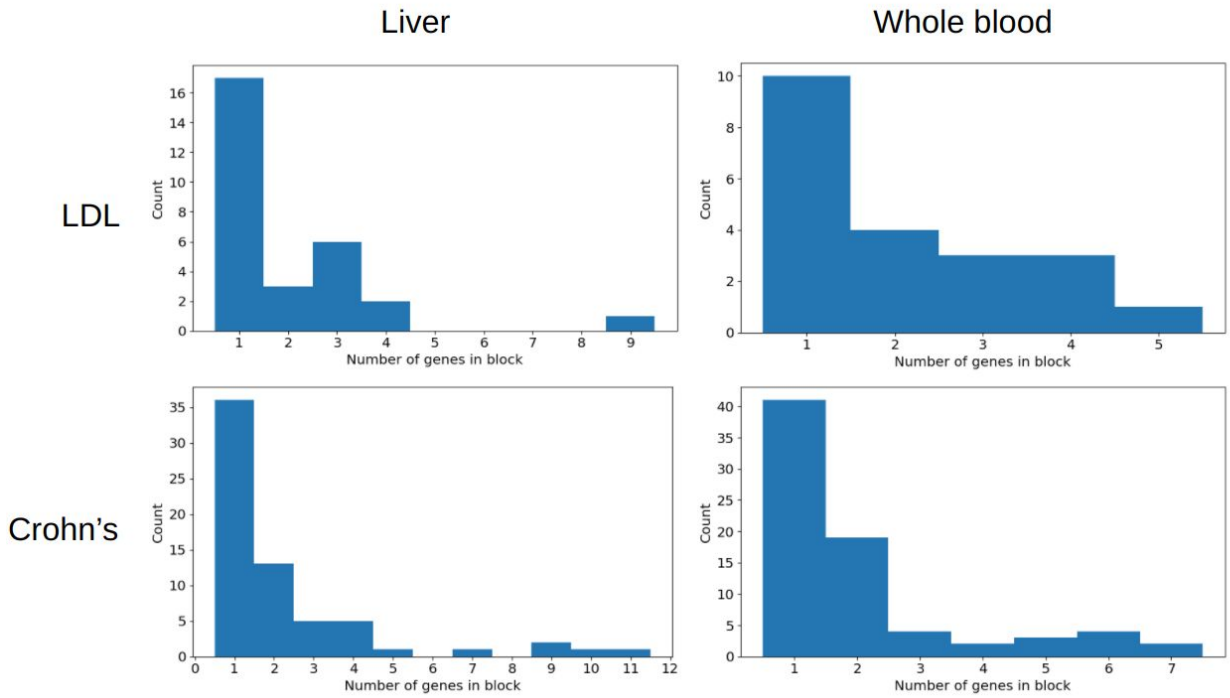

Figure S3: Number of TWAS hit genes per locus after 2.5-MB clumping.

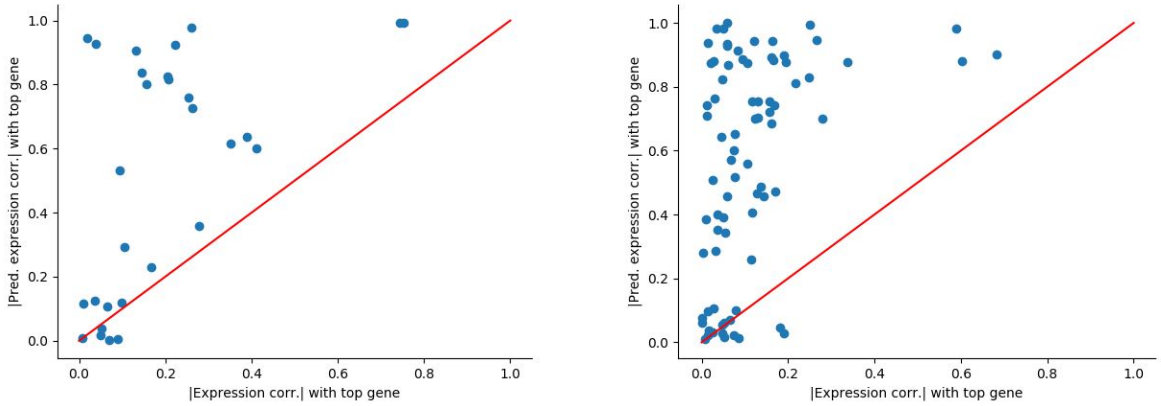

Figure S4: Total versus predicted expression correlation versus the top hit, for all genes in multi-hit blocks that are not the top hits. a) Liver, LDL. b) Crohn's, whole blood. Note that predicted expression correlation is generally higher than total expression correlation, as discussed in the Results section.